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The hepatoprotective role of reduced glutathione and its underlying mechanism in oxaliplatin-induced acute liver injury

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Abstract. Currently, the underlying mechanism of oxaliplatin (OXA) induced live injury is unclear. In addition, there is no standard clinical treatment for OXA-induced acute liver injury (ALI). In this study, we established an animal model of OXA-induced ALI, and studied the role of oxidative stress in OXA-induced ALI and the impacts of reduced glutathione (GSH) treatment on OXA-induced ALI. To establish an OXA-induced ALI model, KM mice received intraperitoneal injection of OXA (8 mg/kg) for 4 days. Serum alanine aminotransferase (ALT), aspartate aminotransferase levels (AST), hepatic pathology and oxidative stress indicators in liver tissues were analyzed. To study the impact of GSH treatment on OXA-induced ALI, mice were treated with GSH (400 mg/kg, i.p). In this ALI mouse model, ALT and AST levels were significantly increased ($P<0.01$). Liver pathological examination revealed varying degrees of liver cell turbidity and degeneration, even balloon-like changes and focal necrosis, and sinusoidal hemorrhage in some cells. Compared with control group, the malondialdehyde (MDA) and GSH levels were significantly increased in OXA-treated group ($P<0.01$), while the superoxide dismutase SOD and GSH-peroxidase levels were decreased after OXA withdrawal ($P<0.01$). When GSH was used to treat OXA-induced ALI mice, the pathological injury of liver tissues was alleviated, and serum ALT and AST were significantly decreased. In addition, GSH treatment could reduce the OXA-induced increase of MDA level ($P<0.05$) in liver tissues, but had no impact on SOD level ($P>0.05$). We have successfully established an OXA-induced ALI model. Using this model, we discover that oxidative stress plays an important role in OXA-induced ALI. GSH-based hepatoprotective therapy can partially inhibit oxidative stress and alleviate OXA-induced ALI.

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Introduction

Oxaliplatin (OXA) is a third-generation platinum compound and OXA-based chemotherapy is a widely used treatment for solid organ malignancies. The combination of OXA with other chemotherapy agents, including 5-fluorouracil/folic acid (FOLFOX) and capecitabine, is a first-line therapy for colorectal cancer (1). Despite its utility, OXA-based chemotherapy is associated with chemotherapy-associated liver injury. Rubbia-Brandt *et al* (2) reported that 78% of patients with metastatic colorectal cancer receiving OXA-based chemotherapy experience varying degrees of sinusoidal injury to the liver. A number of other studies have also suggested that OXA can cause liver injury (2,3). FOLFOX is associated with the development of sinusoidal obstruction syndrome (SOS) and nodular regenerative hyperplasia (3). Soubrane *et al* (4) revealed that liver histopathological changes occur in ~59% of patients who have received OXA-based preoperative chemotherapy followed by hepatic resection for colorectal liver metastases. In addition, OXA-based chemotherapy is associated with increased peri-operative morbidity, including post-hepatectomy liver failure and prolonged prothrombin time (5-7). Furthermore, 10-60% of patients receiving OXA-based chemotherapy have abnormal liver function which can cause chemotherapy delays and necessitate dose reduction, as well as increase the incidence of irregular events during chemotherapy (6,8).

Currently, the underlying mechanism of OXA-induced liver toxicity is unclear. One hypothesis is that OXA-induced liver

damage may be associated with oxidative stress (9-11). In a mouse model of OXA-induced liver injury, Robinson *et al* (10) observed that the expression levels of certain oxidative stress-related genes, including metallothionein 1 (Mt1), heme oxygenase 1 (HO1) and superoxide dismutase 3 (SOD3), were all upregulated. This indicates that oxidative stress may serve a central role in FOLFOX-induced SOS that can be prevented by the administration of the antioxidant butylated hydroxyanisole (10). Schwingel *et al* (11) determined that the antioxidative compounds resveratrol, quercetin (QT) and quercetin nanoemulsion (NQT) can effectively alleviate OXA-induced liver toxicity in a murine model. In addition, several antioxidative compounds can ameliorate steatohepatitis and OXA-induced neurotoxicity through reducing oxidative stress (11-13).

However, prior clinical and animal studies have focused on studying chronic liver injuries caused by long-term use (4-8 weeks) of OXA-based chemotherapy. Currently, few studies are performed using animal models of OXA-induced acute liver injury (ALI). In addition, there are limited reports available regarding the pathological changes in patients with ALI receiving OXA-based chemotherapy. Due to ethical issues and unwillingness of patients to receive a liver needle biopsy, it is difficult to perform clinical studies on OXA-induced ALI.

At present, there is no standard clinical treatment for OXA-induced ALI. Clinicians can only use experience to select one or a combination of various hepatoprotective drugs, one of which is reduced glutathione (GSH). GSH is a bioactive peptide and important non-enzymatic antioxidant widely present in living organisms (14). The highest levels of GSH appear in the liver, which is the major organ for GSH synthesis and metabolism. GSH can promote the metabolism of sugar, fat and protein, and maintain normal cell metabolism and cell membrane integrity. It can bind toxic substances, such as electrophilic radicals and oxygen free radicals, and has extensive antioxidative effects (14). Currently, GSH preparations are widely used for treating certain liver diseases, including viral hepatitis, liver cirrhosis and drug-induced liver injury (14,15). Although GSH is empirically selected for the prevention and treatment of OXA-induced liver injury, the protective role of GSH and its underlying mechanism in OXA-induced ALI remain unclear, and associated studies are rare. Due to the aforementioned challenges, it is often problematic to obtain liver histological specimens from patients with cancer and OXA-induced ALI, which restricts the prospects of studies on OXA-induced ALI associated with hepatoprotective therapies. Therefore, an animal model of OXA-induced ALI was established, in order to study the role of oxidative stress in and the hepatoprotective function of GSH treatment on OXA-induced ALI.

Materials and methods

Ethical statement. All animal studies were performed according to the guidelines of the Chinese Council on Animal Care and were approved by the Affiliated Tumor Hospital of Guangxi Medical University Committees on Animal Experimentation (Nanning, China).

Drugs and reagents. OXA for injection (no. 13092615; Jiangsu Hengrui Medicine Co., Ltd.); alanine aminotransferase (ALT) kit (no. 2014007; Changchu Huli Biotech Co., Ltd.); aspartate aminotransferase (AST) kit, GSH kit, SOD kit, glutathione peroxidase (GSH-px) kit, malondialdehyde (MDA) kit and total protein quantification kit (BCA method) (all 6 kits are no. 20140402; Jiangcheng Bioengineering Institute (Nanjing, China).

In vivo chemotherapy model. Twenty male KM mice (aged 8-10 weeks and weighing 26-28 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). All mice were housed under standardized conditions with one cage for every 5 mice, *ad libitum* access to a standard chow and water, and 1 week to adapt to the laboratory environment prior to manipulation. The room temperature was 22-25˚C with 45-55% humidity and a 12-h light-dark diurnal cycle (lights on between 7:00 a.m. and 7:00 p.m.). Mice were treated with 8 mg/kg OXA (0.5 ml), administered via intraperitoneal injection (i.p.), for 4 days. The drug regimen was based on previously published studies (10,11) and the preliminary dose exploration experiment. Control animals only received 5% glucose (10 ml/kg, i.p.). There were 10 animals per treatment group. Mice were randomly culled by cardiac puncture under isoflurane anesthesia 12 h after OXA injection until the end of the experiment. Mice were anesthetized separately using 2% isoflurane and an incision was made in the middle of the abdomen, prior to samples (blood and liver tissue) being collected for further analysis. The characteristics of the mice (mental state and hair color) and the body weights were examined every day for abnormalities. Pathological examination was performed following hematoxylin and eosin (H&E) staining of the liver tissue sections. To assess the impact of GSH treatment on OXA-induced ALI, mice (n=10 per group) were treated with OXA (10 mg/kg, i.p.) and GSH (400 mg/kg, i.p., 30 min prior to first OXA injection) for 4 days (once daily until the end of the experiment). Mice were euthanized via deep anesthesia with isoflurane 3 days after the final dose of chemotherapy. Samples (blood and liver tissues) were collected for further analysis.

Pathological examination of mouse liver tissues. Liver tissues were fixed in 4% paraformaldehyde, and then embedded in paraffin. After sectioning, the liver specimens were stained with H&E. As observed via optical microscopy, the pathological changes associated with liver injury included liver cell turbidity and degeneration, balloon-like changes and necrosis. According to the coverage of abnormal liver cells, liver injuries were graded as follows: Level 0, normal, no liver cell degeneration; level 1, mild, the ratio of hepatic lobule lesion <1/3; level 2, moderate, the ratio of hepatic lobule lesion was between 1/3 and 2/3; level 3, serious, the ratio of hepatic lobule lesion >2/3 (+++).

Analysis of serum ALT and AST levels. Blood samples from the mice were centrifuged at 300 x g for 8 min at 37˚C, and the supernatants were measured using an alanine aminotransferase (ALT) kit (HuiLi Biotech Co., Ltd., Changchun, China) and an aspartate aminotransferase (AST) kit (Jiancheng Bioengineering Institute, Nanjing, China), according to the manufacturer's protocols. The results are represented as units/l.

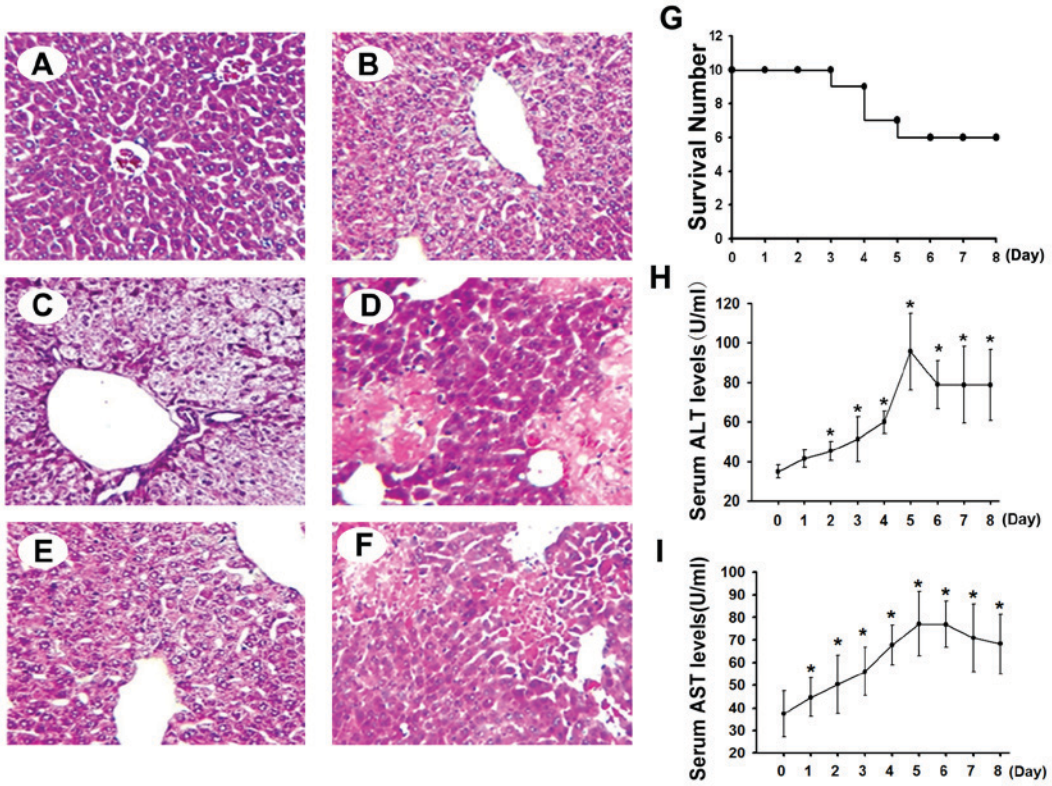

Figure 1. OXA-induce ALI in mice. KM mice were treated with OXA (8 mg/kg, i.p.) for 4 days. Following OXA withdrawal, the mice were observed for 4 days. The mice treated with 5% glucose (i.p.) for 4 days were used as the control group. (A-F) Representative images of the histological evaluation of H&E stained liver tissues (x100). (A) Control liver tissue. (B) Liver cell turbidity and degeneration. (C) Certain liver cells exhibited balloon-like degeneration. (D) Dot-like liver cell necrosis. (E) The liver tissue at 2 days following OXA withdrawal. (F) The liver tissue of deceased mice. (G) The survival rate of the OXA-treated group. (H and I) Time course study of ALT and AST serum levels in the OXA-treated group. Results are presented as the means ± standard deviation from five mice in each group. *P<0.05. OXA, oxaliplatin; ALI, acute liver injury; ALT, alanine aminotransferase; AST, aspartate aminotransferase levels; H&E, hematoxylin and eosin.

Analysis of oxidative stress indicators. Proteins were extracted from whole liver tissues in RIPA buffer and quantified using a Bradford assay (Nanjing Jiangcheng Bioengineering Institute). The GSH, GSH-Px, SOD and MDA content of liver tissues were detected using the kits obtained from the Nanjing Jiangcheng Bioengineering Institute, according to the protocols provided by the manufacturer.

Statistical analysis. All statistical analyses were performed using SPSS version 10 (SPSS, Inc., Chicago, IL, USA). All experiments were performed using 3-5 mice per experimental group and repeated at least three times to assess reproducibility. Differences were analyzed using Student's t-test or one-way analysis of variance, followed by Tukey's post hoc test. Cumulative survival time was calculated using the Kaplan-Meier method and was analyzed by the log-rank test. Data are presented as the mean ± standard deviation. $P<0.05$ was considered to indicate a statistically significant difference.

Results

A mouse model of OXA-induced ALI was successfully established. To establish a mouse model of OXA-induced ALI, KM mice were treated with OXA (i.p.) for 4 days. Following 2 days of OXA treatment, mice exhibited a reduced appetite and mild diarrhea, which were aggravated with an increase in OXA treatment. A number of mice experienced severe diarrhea, and ultimately died. No abnormal pathological changes were observed in the control mice (Fig. 1A), while liver injuries, including mild liver cell swelling, liver cell turbidity and degeneration, and loose cellular structure, were observed following 3 days of OXA treatment in the OXA group (Fig. 1B). Varying degrees of liver cell turbidity and degeneration (Fig. 1C-E), and even balloon-like changes and focal necrosis, were observed in the liver tissues following OXA withdrawal; these liver pathological changes were most evident at 2 days following OXA withdrawal. The major liver pathological changes present in the deceased mice were moderate cell turbidity and degeneration and focal necrosis (Fig. 1F). Survival curve analysis revealed that mortality occurred following 4 days of OXA treatment in the OXA group, and the survival rate in this group was 60% (6/10) 7 days after the final dose of OXA was administered (Fig. 1G).

To evaluate OXA-induced liver toxicity in the mouse model, changes in the serum AST and ALT levels were detected. Compared with the control mice, OXA-treated mice showed significantly elevated serum ALT and AST levels ($P<0.05$) after 2 days and 1 day of OXA treatment, respectively. With the increase in the number of OXA treatments, these elevations were enhanced, and the high serum AST and ALT levels persisted for 4 days following OXA withdrawal (Fig. 1H and I).

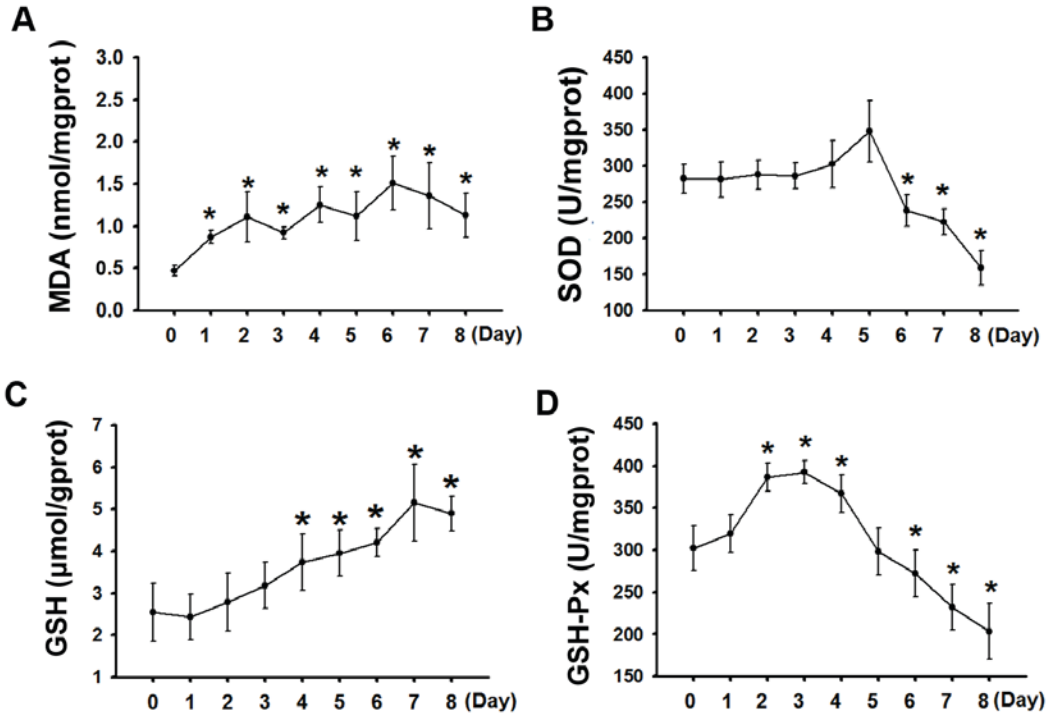

Figure 2. Oxidative stress during OXA-induced hepatotoxicity in the mouse model. Time course study of the levels of the oxidative indicator MDA (A) and the antioxidative indicators SOD (B), GSH (C) and GSH-Px (D) in the liver tissues of OXA-treated mice. Results are presented as the means ± standard deviation from five mice in each group. *P<0.05. OXA, oxaliplatin; GSH-Px, glutathione peroxidase; MDA, malondialdehyde; ALT, alanine aminotransferase; AST, aspartate aminotransferase levels; SOD, superoxide dismutase.

Oxidative stress in OXA-induced ALI. Evidence from various patient studies suggests that liver injuries induced by OXA-based chemotherapy, including FOLFOX-induced SOS, are associated with increased oxidative stress in the liver (10). To elucidate the role of oxidative stress in OXA-induced ALI, the oxidative indicator MDA and the antioxidative indicators SOD, GSH and GSH-Px, were analyzed. As presented in Fig. 2A, the MDA levels in OXA-treated mice were significantly increased 1 day following OXA injection (P<0.05), a difference that was enhanced as the OXA injection dose increased (P>0.05). MDA was maintained at high levels even several days following the termination of OXA treatment. Compared with the control group, no significant change in SOD levels was observed during OXA treatment, but decreased SOD levels were observed 2 days following OXA withdrawal (P<0.05; Fig. 2B). GSH levels did not significantly change during early OXA treatment (P>0.05), but continuously increased during later OXA treatment and the early period following OXA withdrawal (P<0.05; Fig. 2C). In OXA-treated mice, GSH-Px levels were significantly increased following OXA injection (P<0.05; Fig. 2D), but was decreased 2 days following OXA withdrawal and thereafter remained at low levels.

GSH attenuates OXA-induced ALI. To examine whether GSH therapy has a protective effect on OXA-induced ALI, OXA-treated mice received GSH treatment 30 min prior to each OXA injection for 4 days. Optical microscopy and H&E staining indicated clear liver cell injury in OXA-treated mice, including liver cell swelling and degeneration (mainly moderate and severe), balloon-like changes and focal necrosis (Fig. 3A). Compared with the OXA group mice, GSH group mice exhibited alleviated liver cell injury, which demonstrated mild turbidity and swelling, and no notable hepatocyte necrosis (Fig. 3A). In addition, the serum AST and ALT levels in the GSH group mice were markedly decreased, compared with those in the OXA group mice (P<0.05; 46.77±7.64 vs. 72.17±15.34, 42.37±15.83 vs. 60.78±24.94 for ALT and AST, respectively), but were still higher than those in the control mice (P<0.05; Fig. 3B and C). However, in the GSH-treated group, GSH did not significantly alleviate the OXA-induced reduced appetite, decreased body weight and diarrhea (data not presented). Body weight increased over time in the control mice, but significantly decreased in the OXA and GSH groups (P<0.05). There was no significant difference between the OXA group and GSH group (P>0.05) with respect to body weight (Fig. 3D). In addition, GSH therapy did not increase the survival rate of the GSH group (Fig. 3E) compared with the OXA group (60 vs. 60%).

GSH suppresses OXA-induced oxidative liver injury. The antioxidative effect of GSH on liver injury was investigated. As presented in Fig. 4A and B, GSH administration decreased the liver MDA and GSH levels in the GSH group, compared with the OXA group (P<0.05; 0.43±0.12 vs. 1.23±0.50, 3.77±1.25 vs. 4.87±0.64 for MDA and GSH, respectively). Compared with in the control group, liver GSH-PX activity was significantly decreased (P<0.01) in the OXA group, and this was reversed by GSH administration (Fig. 4C). However, no significant difference in SOD activity was observed between the OXA and GSH groups (Fig. 4D).

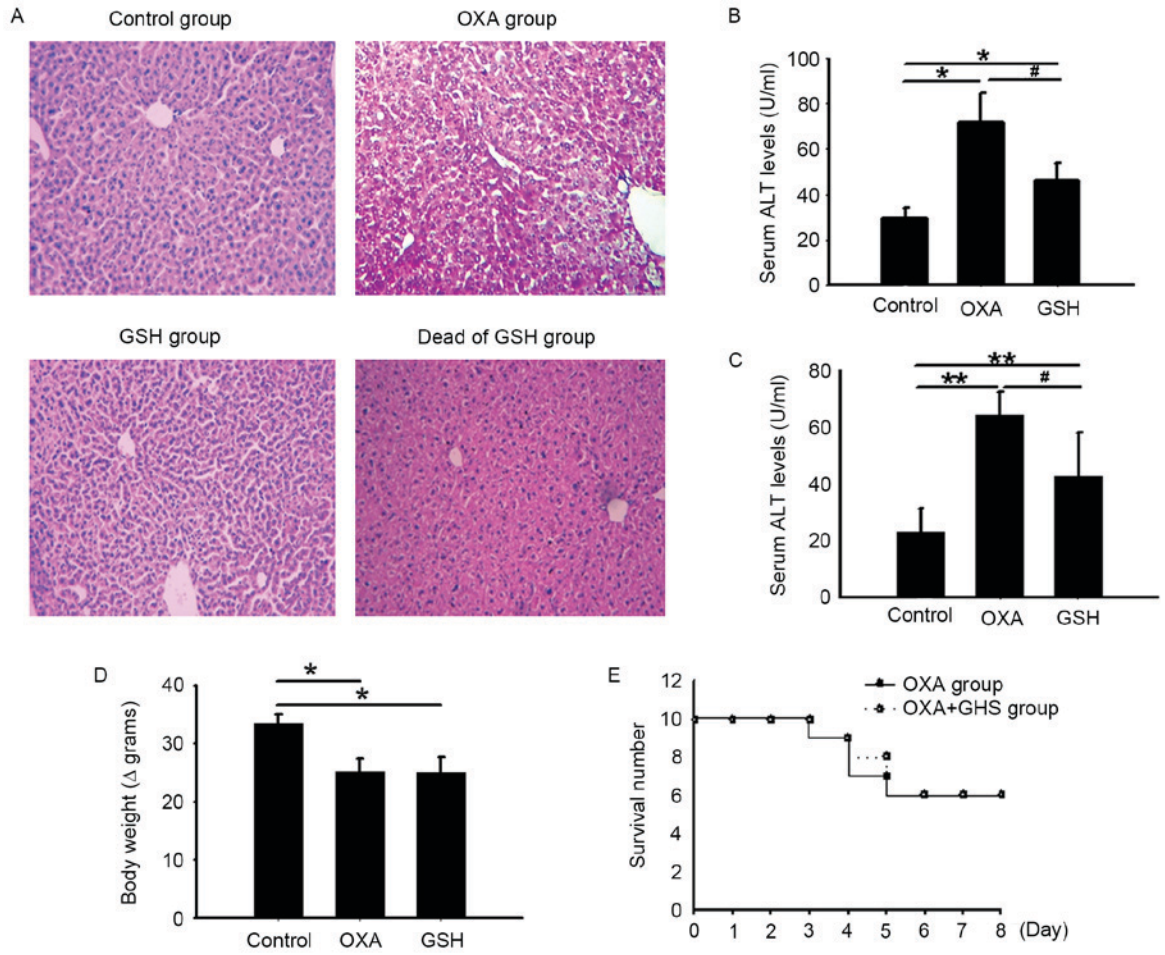

Figure 3. Treatment with GSH attenuated OXA-induced ALI in mice. The OXA group were treated with OXA for 4 days, the GSH group were treated with OXA for 4 days and with GSH every day from the first day of OXA administration until the end of the experiment, and the control group were administered with 5% glucose (i.p.) for 4 days. The samples (blood and liver tissue) from each group were collected 3 days after the final dose of OXA. (A) The liver histopathology was examined in each group (H&E staining, original magnification, x100). (B) The serum ALT and AST levels of each group 3 days after the final dose of OXA. (C) The body weights of each group 3 days after the final dose of OXA. For (B) and (C), the results are presented as the means ± standard deviation from five mice in each group. *P<0.05 and **P<0.01, compared with the control group. $^{\#}$P<0.05, compared with the OXA group. (D) The survival rates of the three groups were observed. OXA, oxaliplatin; GSH, glutathione; ALI, acute liver injury; ALT, alanine aminotransferase; AST, aspartate aminotransferase levels; H&E, hematoxylin and eosin.

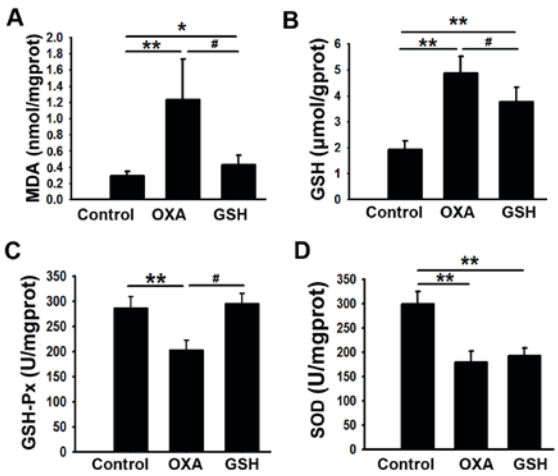

Figure 4. GSH treatment suppressed OXA-induced oxidative stress. (A) MDA, (B) GSH, (C) GSH-Px and (D) SOD in the liver tissues of the OXA, GSH and control groups. The results presented are the mean ± standard deviation of three independent experiments performed in triplicate. *P<0.05 and **P<0.01, compared with the control group. $^{\#}$P<0.05, compared with the OXA group. OXA, oxaliplatin; GSH-Px, glutathione peroxidase; SOD, superoxide dismutase; MDA, malondialdehyde.

Discussion

Chemotherapy-associated liver injury can include steatosis, liver cell necrosis, severe steatohepatitis and SOS. Distinct types of liver injuries may be associated with specific chemotherapy drugs (16,17). In patients with colon cancer receiving multi-cycle OXA-based chemotherapy, liver injury pathological changes include steatosis and sinusoidal injury, in addition to elevated AST and phosphatase levels (6). SOS is the most typical histological change, and is characterized by impaired sinusoidal wall integrity, sinusoidal hyperemia and blockage, sinusoidal fibrosis, fibroid blockage in the lobular central vein and nodular hyperplasia or hemacelinosis (18). Similar pathological changes to the liver are also observed in animal models of OXA or OXA-based chemotherapies. Schwingel *et al* (11) treated BALB/c mice with OXA (10 mg/kg/week, i.p.), and reported the appearance of steatohepatitis after 6 weeks of OXA treatment. Keizman *et al* (19) treated C57BL/6 mice with OXA (10 mg/kg/week, i.p.) for 4 weeks, and established a mouse model of OXA-induced steatohepatitis. Robinson *et al* (10) treated mice with FOLFOX

(10 mg/kg/week, i.p.) for 6 weeks, and successfully established an animal model of OXA-induced SOS.

A mouse model of OXA-induced ALI was successfully established in the current study. In this model, elevated ALT and AST levels characterized OXA-induced ALI during the early stage of OXA treatment. Hepatic histopathology of the OXA-induced ALI demonstrated varying degrees of liver cell turbidity and degeneration, even balloon like changes and focal necrosis, and sinusoidal hemorrhage in certain individuals. These hepatic pathological changes in OXA-induced ALI were different from the pathology of chronic liver injuries induced by multi-cycle OXA-based chemotherapy reported in clinical observation and animal studies, in which the primary characteristics of liver injury are liver sinusoidal injury and SOS (2,3). Therefore, liver sinusoidal injury and SOS are the pathological characteristics of long-term OXA chemotherapy (18), while OXA-induced ALI is characterized by varying degrees of liver cell degeneration, such as turbidity-like degeneration and balloon-like degeneration.

Recently, it has been suggested that oxidative stress is an important contributing factor to hepatotoxicity induced by long-term OXA chemotherapy (9,10). Oxidative stress is the overproduction of highly active molecules, such as ROS, and when liver cells are exposed to certain noxious stimuli, leading to an imbalance between the oxidative and antioxidative systems, liver injury occurs (20). In the present study, it was revealed that the level of oxidative indicator MDA is increased in OXA-treated mice. MDA is a lipid peroxidation product, and its level can reflect the extent of oxidative stress-associated injury caused by free radicals (21). In the OXA-induced ALI model, elevated MDA levels indicate that OXA can increase free radicals in the liver. Excessive MDA in liver tissue will consume a large amount of antioxidative factors, such as SOD and GSH, which can protect liver from the attacks of free radicals, but once the balance is broken, SOD and GSH will be unable to protect liver against the excessively increased MDA (22,23). The present study demonstrated that, although GSH levels are continuously increased following OXA withdrawal and liver MDA levels are continuously increased, GSH-Px and SOD levels are consistently decreased and are accompanied by elevated ALT and AST levels. Additionally, pathological examination of the liver revealed an increase in liver injury following OXA administration. Furthermore, an increase in mouse mortality was also observed following an increase in the number of OXA treatments. These results indicate that the OXA-induced increase in liver free radicals, massive depletion of SOD and the insufficient compensation of GSH-Px and GSH syntheses all lead to the occurrence of ALI. Therefore, the results suggest that oxidative stress may serve an important role in the pathogenesis of OXA-induce ALI.

Under physiological conditions, the liver can resist oxidative stress through GSH synthesis in hepatocytes. In the present study, mice treated with OXA and GSH exhibited high GSH-Px levels and low MDA levels, which indicated a reduction of oxidative stress and is accompanied by decreased tissue injury, ALT and AST levels. GSH can directly scavenge radicals and peroxides via mixed disulfide formation or oxidization to generate oxidized glutathione (14-15,24). GSH can resist oxidative stress by serving as a substrate for antioxidative enzymes, including GSH-Px which converts hydroperoxide into less harmful fatty acids, water and GSH disulfide (24). Therefore, GSH can resist OXA-induced oxidative stress, and attenuate OXA-induced liver injury.

In the present study, MAD levels in the GSH treatment group remained higher than in the control group, and no significant impact on SOD level downregulation was observed following GSH treatment. Therefore, although GSH treatment exerted a significant protective effect against OXA-induced liver injury in the present study, hepatic oxidative stress continues to occur. In addition, the ALT and AST levels in OXA and GSH-treated mice did not recover to within the normal range, indicating that GSH alone is insufficient for suppressing oxidative stress during OXA-induced ALI. Perhaps combining GSH with other drugs, such as antioxidants, may further alleviate OXA-induced liver injury. Indeed, various endogenous of dietary antioxidants are capable of ameliorating steatohepatitis and OXA-induced neurotoxicity via reducing oxidative stress. Besides oxidative stress, prior studies determined that other mechanisms are also involved in OXA-induced liver injury. These mechanisms include the activation of inflammation-associated pathways (10,25,26), the activation of cellular hypoxia (27) and the upregulation of genes involved in coagulation (particularly PAI-1 and vWF) (3,10,28). Studies have also detected the upregulation of angiogenesis-associated genes, including VEGF-A, VEGF-C and VEGF-D in OXA-induced SOS (10,27,29). Concordantly, prior clinical observations suggested that bevacizumab is effective in reducing the incidence and severity of SOS associated with OXA-based chemotherapy (28,30,31). Therefore, to further alleviate OXA-induced liver injury, it is essential to consider other potential mechanisms that contribute to liver injury, which will be examined in subsequent studies.

As observed in the present study, GSH treatment alone cannot reduce OXA-induced mortality. Histopathological examination detected no liver failure, and the cause of mortality was determined to be severe diarrhea. Compared with the OXA-treated mice, OXA and GSH-treated mice exhibited no significant difference in body weight loss, appetite reduction and diarrhea (data not presented), indicating that GSH treatment has no significant ameliorative effect on OXA-induced liver injury. Therefore, during treatment of the liver injury caused by OXA chemotherapy, other OXA-induced toxicities, including neurotoxicity, gastrointestinal toxicity and hematological toxicity, must also be considered.

In summary, an animal model of OXA-induced ALI was successfully established. The results suggest that oxidative stress serves an important role in the pathogenesis of OXA-induced ALI, and that GSH treatment can attenuate OXA-induced ALI by suppressing oxidative stress in the liver.

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